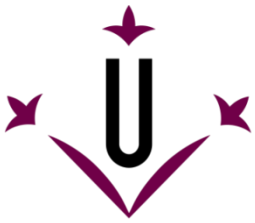

Fate of the mycotoxins in the wort and yeast during ale and lager fermentation and their evaluation under different technological parameters

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ABSTRACT

This study aimed to evaluate the mycotoxins transfer in the wort and yeast during ale and lager fermentation, secondary fermentation, and beer storage**.** Four yeasts were used to ferment wort contaminated with DON and ZEN. The wort was fermented with two *S. cerevisiae* yeasts (yeasts A and B) at 15, 20, 25 °C for 96 h, and two *S. pastorianus (yeasts C and D)* at 10, 15, 20 °C for 144 h. After fermentation, the two wort with the highest mycotoxin decrease were selected for a second fermentation and stored period. Mycotoxins were extracted using QuEChERS and analyzed by UHPLC-DAD/FLD. In the wort, DON decreased from 11 % to 27 %, locating it adsorbed on the yeast from LOD to 6.16 %. ZEN in the wort decreased from 29 % to 90 %, locating it adsorbed on the yeast from 5.41 % to 33.86 %. Ale-fermentation process obtained the highest decrease of ZEN in the wort, whereas, for DON, there was no significant difference in the reduction between the 2 fermentation styles. Finally, there was no significant change in mycotoxin content during storage. Knowledge of the mitigating effect of fermentation variables could help to reduce the mycotoxin content in beer.

Key words: Deoxynivalenol; zearalenone; *Saccharomyces* yeast; Secondary fermentation; Beer

1. Introduction

The mycotoxins produced by *Fusarium* species are characterized by a high chemical and physical stability and are not eliminated by technological treatments, thus a transfer from raw materials to beer is possible (Rodríguez-Carrasco, Fattore, Albrizio, Berrada, & Mañes, 2015). Mycotoxin detoxification is typically achieved by removal or elimination of the contaminated elements or by total or partial inactivation of the toxins present in these products. In addition to physical and chemical methods, biological methods have proved to be an efficient option for the detoxification of mycotoxins (Vila-Donat, Marín, Sanchis, & Ramos, 2018).

Recent surveys have shown the presence of low concentrations of mycotoxins, mainly deoxynivalenol (DON), deoxynivalenol-3-glucoside(DON-3-Glc), zearalenone (ZEN) and fumonisins (FBs) in commercial beers from different countries (Pascari, Ortiz-Solá, Marín, Ramos, & Sanchis, 2018; Peters et al., 2017; Wall-Martínez, Pascari, Ramos, Marín, & Sanchis, 2019). Our research group has analyzed the fate of *Fusarium* mycotoxins during beer production processes such as malting (Pascari, Gil-Samarra, Marín, Ramos, & Sanchis, 2019) and mashing (Pascari, Rodriguez-Carrasco, et al., 2019), (i) identifying its decrease during steeping, (ii) confirming its conversion to DON-3-Glc during germinationand (iii) its transfer to the wort after the first stage of the mashing process.

Beer fermentation process is initiated by two main yeast strains *S. cerevisiae,* in the case of ale or top-fermented beers and *S. pastorianus,* in the case of lager or bottom-fermented beers. Their metabolic activity is possible between 5 and 30 ºC. Sometimes, in the search for a more unique flavour of the beer, the brewer might decide to perform a secondary fermentation. This is usually the case for lager beers, and from the technological perspective, it is a difficult task as it implies a higher risk of contamination with altering bacteria (Kunze, 2006).

Wall-Martínez, Pascari, Bigordà, et al. (2019) evaluated the effect of 15 commercial yeasts (*S. cerevisiae and S. pastorianus*) in the mitigation of DON and ZEN during beer fermentation at 20 °C for all the yeast strains tested and at a single mycotoxin concentration. *Fusarium* mycotoxins were extracted with immunoaffinity columns specific to each mycotoxin and analysed with UHPLC-FLD/DAD. At the end of the fermentation process, 10–17 % of DON and 30–70 % of ZEN were removed, of which up to 6 % of

the initial concentration of DON and 31 % of the ZEN were adsorbed to the yeast cell. Garda et al. (2005) reported a reduction of 53% in malt spiked with DON; however, Nathanail et al. (2016) observed a 15% reduction of DON content in the wort at the end of the alcoholic fermentation. The wide variation between the results obtained in the three studies could be due to the use of different fermentation parameters, mycotoxin content, or yeast strains that could have had different adsorption potential.

The increasing awareness of the food industry regarding the mycotoxins shows the need of developing analytical methods that are faster, greener, more precise, and guarantee the quality, authenticity, safety, and traceability of target compound in a diversity of sample matrices (Souza-Silva, Gionfriddo, & Pawliszyn, 2015). In this context, QuEChERS method is one of the most promising and user-friendly during the mycotoxin extraction, involving reduced sample amounts and organic solvents (Perestrelo et al., 2019). This study aimed to evaluate the fate of DON and ZEN in the wort and the yeast during ale and lager fermentation, secondary fermentation, and storage using the QuEChERS method. Also, their fate under different technological parameters was evaluated.

2. Materials and methods

2.1 Reagents and chemical

Water was obtained from a Milli-Q® Reagent from Millipore Corp (Brussels, Belgium). Methanol and acetonitrile were purchased from Scharlab (Sentmenat, Spain). Mycotoxin standards were purchased from Romer Labs (Tulln, Austria). Immunoaffinity columns for DON and ZEN were purchased from R-Biopharm (Glasgow, UK). C18 was purchased from Phenomenex (Torrance, USA), Magnesium sulfate heptahydrate was acquired from Probus SA (Badalona, Spain) and agar powder from VWR Prolabo (Leicestershire, UK), PBS was prepared with potassium chloride (0.2 g) (Panreac, Castellar del Vallès, Spain), potassium dihydrogen phosphate (0.2 g) (Castellar del Vallès, Spain), disodium phosphate anhydrous (1.16 g) (Castellar del Vallès, Spain) and sodium chloride (8.0 g) (Fisher Bioreagents, New Jersey, USA) in 1 L of Milli-Q water. YEPD was prepared with 10 g/L of yeast extract powder (Bacto, Madrid, Spain), 20 g/L of peptone, 20 g/L of glucose (Fisher chemical) and 20 g/L of agar.Yeasts were purchased from www.cervezasdelmundo.com.

2.2 Preparation of mycotoxin solutions

DON and ZEN concentration in the stock solution was checked by UV spectroscopy according to AOAC, Chapter 49. Standard solutions of DON and ZEN were prepared in methanol at a concentration of 10 mg/mL and stored at 4 °C. Calibration curves were prepared by appropriate dilution of the stock solution with the mobile phase.

2.3 DON and ZEN contamination of malted barley

Barley (*Hordeum vulgare*) was supplied by a malting plant (La Moravia, Spain) in September 2018. The absence of DON and ZEN in the malt was verified by UHPLC-DAD/FLD. A control batch of malted barley was used to prepare control wort (absence of DON and ZEN); while, another malt batch was contaminated using a toxigenic strain of *Fusarium graminearum* (F.46) obtained from the collection of strains of the Food Technology Department of the University of Lleida, Spain. The grains were disinfected according to Andrews et al. (1997). Briefly, 500 g of grains was submerged into 0.4 g/100 mL sodium hypochlorite solution for 2 min and then abundantly rinsed twice with sterile distilled water. Then, the grains were placed in hermetically closed sterile ISO bottles and left overnight at 4 °C with a small amount of water to allow the water activity to reach a value close to 0.99 (Aqualab Series, Washington, USA). Malted barley was transferred to Petri dishes and 1 mL of a spore suspension of *F. graminearum* was transferred to each dish. Petri dishes were incubated at 25 °C for 30 days. The final mycotoxin concentration in the contaminated malt was 4600 µg/kg and 2800 µg/kg for DON and ZEN, respectively.

2.4 Yeast

Four freeze-dried yeasts strains of various commercial brands, 2 *Saccharomyces cerevisiae* (ale fermentation, code A and B) and 2 *Saccharomyces pastorianus* (lager fermentation, code C and D), who presented the highest adsorption of mycotoxins in study by Wall-Martínez, Pascari, Bigordà, et al. (2019) were used to ferment the wort.

2.5 Wort production

Three batches of wort with different mycotoxin concentration were made. Control wort (prepared from uncontaminated malt), the low mycotoxin contaminated, and the high mycotoxin contaminated.

Contaminated batches were made by mixing different portions of contaminated and uncontaminated malted grain. Maceration was carried out by mixing 2.5 L of deionized water with 500 g of malted barley (previously coarse ground) and then maintaining the mix for 15 min at 45 ºC, followed by 60 min at 65 ºC, ending with 72 ºC for another 15 min. The wort was decanted, the density was adjusted to 1005-1010 kg/m^3 with deionized water and the hop was added (10g/L). The wort was boiled for approximately 2 h (up to a density of 1050 kg/m^3) and stored in sterile bottles until fermentation.

2.6 Primary fermentation, secondary fermentation and storage

First fermentation assays were performed at 250 mL scale. To each yeast, three fermentation temperatures were used:15, 20, and 25 °C for high-ale fermentation using *S. cerevisiae* and 10, 15, and 20 °C for low-lager fermentation using *S. pastorianus*. Yeast concentration was prepared at 10^6 CFU/mL for all the wort samples, initial count was verified using a Thoma cell counter chamber. Sampling was performed daily in the following time points: 24, 48, 72, and 96 h for fermented wort with *S. cerevisiae* and 24, 48, 72, 96, 120 and 144 h for fermented wort with *S. pastorianus.* The two treatments of each yeast species (ale or lager) with the highest mycotoxin reduction in the wort were selected for a second fermentation. The double or second fermentation was carried out at 20°C samples being taken at the end of the process (96 h) in both wort and yeast. The double fermented wort was distributed in 4 bottles of 50 mL for the storage analysis. Every week a 30 mL sample was collected from each bottle for 4 weeks, and the remainder wort was discarded.

Control wort was fermented for all temperatures and yeasts included in the research for the first and second fermentation. At each sampling point, three samples were collected. The first sample (10 mL) was used to determine the pH and alcohol content (portable densimeter DMA, Anton Paar). The second sample (30 mL) was used to determine mycotoxin content and the third sample (1 mL) was diluted from 10^{-1} to 10^{-8} with PBS. Then 100 µL of 10^{-6}, 10^{-7} and 10^{-8} dilutions was superficially spread on YEPD agar medium dishes and incubated at 25 °C. After 48 h the yeast growth was calculated. At the end of every fermentation, yeast was separated from the remaining wort by centrifugation at 1869 x g for 10 min, lyophilized, and weighted. All samples were stored at -18 ºC until UHPLC analysis.

2.7 DON and ZEN extraction in malt

Extraction in malt was performed following R-Biopharm instructions. For DON, five grams of ground sample was mixed with 1 g of sodium chloride and 40 mL of Mili Q water followed by 30 min stirring. Then, samples were centrifuged for 10 min at 1846 x g. Supernatant was filtered through a glass microfiber paper filter (Whatman, Maidstone, UK) and 2 mL of the filtrate was passed through the column. The immunoaffinity column was then washed with 10 mL of bi-distilled water and the toxins were eluted with 3 mL HPLC-grade methanol (1.5 mL performing back-flushing and 1.5 mL for the final elution).

For ZEN, five grams of ground sample was mixed with 25 mL of extraction solvent acetonitrile: distillated water (75:25, *v/v*) and stirred for 30 min. Samples were centrifuged for 10 min at 1846 x g and 10 mL of supernatant was mixed with 40 mL of PBS at pH of 7.4 The obtained 50 mL was passed through the immunoaffinity column which was afterwards washed with 20 mL of PBS. ZEN was eluted with 3 mL of acetonitrile (1.5 mL performing back-flushing and 1.5 mL for the definite elution).

2.8 QuEChERS extraction to beer samples

DON and ZEN extraction in fermented wort was processed as Rodríguez-Carrasco et al. (2015) with slight modifications. Briefly, 6 mL of beer sample was added to 14 mL of acetonitrile and vigorously shaken for 60 s prior the addition of 4 g of anhydrous $MgSO_4$ and 1 g NaCl after which it was agitated at 1846 x g in an orbital rotary shaker (Infors AG CH-4103, Bottmingen, Switzerland) after 30 min the mixture was centrifugated for 8 min at 1869 x g. Then, the acetonitrile extract was submitted to a dispersive solid phase extraction into a tube containing 900 mg $MgSO_4$ and 300 mg C18, it was vortexed for 1 min and centrifuged for 8 min at 1846 x g.

2.9 DON and ZEN extraction in yeast

The DON and ZEN extraction in yeast was performed according to the method described by Campagnollo et al., 2015. Two hundred milligrams of lyophilized yeast were suspended in 2 mL of 0.1M potassium phosphate buffer pH 6.5, mixed on a rotating shaker (Bottmingen, Switzerland) for 60 min at 25 °C and sonicated for 15 min (Brason 2800). The samples were centrifuged (Eppendorf, Hamburg, Germany) at 1846 x g for 10 min and 0.8 mL of the supernatant was removed and analyzed by UHPLC. Control samples (200 mg of yeast in buffer solution) were also prepared and analyzed.

2.10 Sample preparation.

All extracted samples (except yeast extracts) were evaporated under a low nitrogen stream at 40 °C, extracts were resuspended in 1 mL of mobile phase. The obtained extract was filtered with nylon filter (0.4 µm) before being injected (50 µL) to the UHPLC DAD/FLD. All the samples were analyzed by triplicate.

2.11 UHPLC system

An Agilent Technologies 1260 Infinity UHPLC system (California, USA) coupled with an Agilent 1260 Infinity II Diode Array Detector (DAD) and Agilent 1260 Infinity Fluorescence Detector (FLD) was used. Separation was achieved on a Gemini® C18 column from Phenomenex 150x4.6 mm, 5 µm, 110 Å (California, USA). For DON analysis, the absorption wavelength was setup at 220 nm. The mobile phase was composed of methanol:acetonitrile:water Mili Q (5:5:90, *v/v/v*) and set at a flow rate of 1 mL/min. ZEN detection and quantification was performed at 274 nm and 455 nm excitation and emission wavelengths, respectively. The mobile phase was acetonitrile:Milli Q water (60:40,*v:v*) with pH adjusted at 3.2 with glacial acetic acid. Flow rate was set at 0.6 mL/min.

The column temperature was 40 °C, the injection volume was 50 µL and total run time was 20 min for the analysis of both DON and ZEN.

2.12 Validation of analytical methods

Selectivity was checked by injecting 50 µL of standard solution for at least three times, comparing retention time and peak resolution between injections. For linearity check, a calibration curve of eight concentration levels for each toxin (20, 30, 50, 100, 250, 500, 1000, 3000 µg/L for DON and 30, 50, 100, 300, 500, 1000, 1500, 3000 µg/L of ZEN solutions) was prepared and injected into the system, generating a linear regression plotting solutions concentration versus peak area.

Precision was evaluated preparing blank wort samples spiked µg/kg with DON and ZEN at concentration levels (75, 250 and 1000 µg/kg) and percentages recovery were determined. The validation parameters are shown in Table 1.

The recovery percentages using the QuEChERS method were similar to those made in previous works (Wall-Martínez, Pascari, Bigordà, et al., 2019) using immunoaffinity columns and the same HPLC system. The RSD was below 10%.

The recovery and relative standard deviation levels in yeast were previously described in Wall-Martínez, Pascari, Bigordà, et al. (2019) 67–71 % for DON and 73–80 % for ZEN. The limit of detection (LOD) was considered as three times the signal to noise ratio. Method performance was assessed according to Commission Regulation (EC) 401/2006 (European Commission, 2006).

2.13 Statistical Analysis

All the experiments results were represented as the average of triplicate tests and expressed as mean ± standard deviation. Statistical evaluations were performed by one-way analysis of variance (ANOVA) and Tukey test (p = 0.05) using Minitab 18 software.

3. Results and discussion

3.1 Characteristics of the wort

Relative density and pH of the wort were 1.040 g/cm^3 and 4.96, respectively. Mycotoxin contamination was 1164 ± 57 µg/kg for DON and 440 ± 16 µg/kg for ZEN in high contaminated wort and 560 ± 49 µg/kg for DON and 284 ±14 µg/kg for ZEN in low contaminated wort. DON and ZEN levels in control wort were below the detection limits.

3.2 Alcohol produced

The DON and ZEN concentration in wort had no significant effect on the pH, yeast growth or alcohol produced. It is known that temperature affects the population dynamics of *Saccharomyces* strains during alcoholic fermentation (Charoenchai, Fleet, & Henschke, 1998; Torija, Rozès, Poblet, Guillamón, & Mas, 2003). The growth rate of yeasts was 3 logarithmic units, except for the treatments with lower fermentation temperatures (15 °C for S. *cerevisiae* and 10ºC for S. *pastorianus*), which was of 2 logarithmic units, decreasing the alcohol production in the wort at the end of the fermentation.

The results of the present work are aligned with the published literature confirming that the presence of mycotoxins in the wort did not have any impact on the fermentation process compared to the control sample.

The previous results suggest that beer producers should pay primary attention to the content of mycotoxins in the raw material since mycotoxin contamination in the wort does not affect the physicochemical characteristics regularly measured (alcohol content, pH, growth of yeasts) in the final product.

3.3 Effect of temperature and mycotoxin concentration in DON reduction during primary fermentation

The fate of DON during beer fermentation using *S. cerevisiae* ale and *S. pastorianus* lager yeasts is shown in Table 2.

DON content decreased between 11 % and 27 % in fermented wort with *S. cerevisiae* and from 14 % to 25 % in fermented wort with *S. pastorianus,* reaching a higher decrease than Nathanail et al. (2016) (15 %) and Wall-Martínez, Pascari, Bigordà, et al. (2019) (from 2 % to 17.5 %) have reported. The mycotoxin concentration in wort had no influence on DON rate reduction. The higher temperatures of fermentation (25 °C and 20 °C for *S. cerevisiae* and 20 °C and 15 °C for *S. pastorianus*) were characterized by the higher rate of DON adsorption to the yeast. This could be attributed to the low growth rate of *Saccharomyces* yeast metabolism at low fermentation temperatures (Charoenchai et al., 1998), reducing the biodegradative effect and the adsorption sites actives of β-glucans from the cell walls (Huwig, Freimund, Käppeli, & Dutler, 2001; Shetty & Jespersen, 2006).

Both yeast strains of *S. cerevisiae* (A and B codes) showed approximately 3 % of initial DON content adsorbed at the yeast cell but only at the higher temperatures (20 and 25ºC). It could be explained by a higher cell mass accumulated during the fermentation process at these temperatures compared to the lower temperatures. *S. pastorianus* (codes C and D) registered an absorption of 4 to 6 % of DON to the yeast cells. This result was achieved at both 15 ºC and 20 ºC in the case of the yeast C, and only at 20 ºC in the case when yeast D was used. The highest adsorption of DON in yeast after fermentation was detected using *S. pastorianus* at 20 °C (yeast C), moreover, this treatment was the only condition where

DON was detected (34.44 ± 1.34 µg/kg) in wort with a low initial level of mycotoxin, this could be because DON adsorption of other treatments would be below LOD (20 µg/kg). Furthermore, it can be seen from Table 2 that the total concentration that was identified in the beer and in the yeast residues suggests that approximately 20% of toxin could be biodegraded to modified DON forms, mainly, DON3G, 3ADON, or 15ADON (Lancova et al., 2008). Also, these mycotoxins forms have been detected in recent beer surveys (Pascari et al., 2018; Peters et al., 2017; Wall-Martínez, Pascari, Ramos, et al., 2019). Nonetheless, the available analytical technique did not allow us to confirm this statement.

3.4 Effect of temperature and mycotoxin concentration in ZEN reduction during primary fermentation

The fate of ZEN during beer fermentation using *Saccharomyces cerevisiae* ale and *Saccharomyces pastorianus* lager yeasts is shown in Table 3.

The decrease of ZEN was between 29 % and 90 % in fermented wort with *S. cerevisiae* and between 39 % and 67 % for fermented wort with *S. pastorianus*, reaching a higher decrease than the level reported by Wall Martínez et al. (2019) (31-72 %). As in the case of DON, higher fermentation temperatures (25 °C for *S. cerevisiae* and 20 °C and 15 °C for *S. pastorianus*) favored ZEN reduction in the wort, and this process conditions led to the highest concentrations of ZEN in the yeast at the end of fermentation. Unlike DON, ZEN was detected in yeasts at the end of fermentation on high and low levels of mycotoxin in the contaminated wort, this may be because the LOD for ZEN is lower than for DON. Similar to Wall-Martínez et al. (2019) with 4 to 31 % ZEN retained by *S. cerevisiae* and 7.5-15 % of ZEN retained by *S. pastorianus*; however, relatively different from the values reported by Campagnollo et al. (2015) (75% of binding in *S. cerevisiae in vitro* test), ZEN was detected from 5 to 33% in *S. cerevisiae* and from 10 to 25 % in *S. pastorianus* yeast. According to the fermentation parameters used there was between 20 and 50 % of ZEN that was not recovered in wort or yeast. It would be relevant to determine the co-occurrence of some masked mycotoxins such as β-zearalenol (β-ZEL), α-zearalenol (α-ZEL) to identify if fermentation is an appropriate process for mitigation of ZEN or if it was biodegraded or metabolized to a form modified as suggested by several authors (Keller et al., 2015; Mizutani, Nagatomi, & Mochizuki, 2011).

3.5 DON and ZEN reduction during secondary fermentation and storage

The mycotoxin content in fermented beer and yeast during double fermentation and beer storage is shown in Table 4.

This is the first study of mycotoxin analysis during secondary fermentation and storage. At the end of the second fermentation, mycotoxin content in the wort was reduced in proportions like the first fermentation (15 % for DON and 44-50 % for ZEN). The mycotoxin adsorbed by the yeast was also in quantities similar to the first fermentation (5% of DON and 21-27 % of ZEN). On the other hand, no significant difference was found in mycotoxins content of the double fermented wort after four weeks of storage. The total reduction of mycotoxins including primary, secondary fermentation, and storage were between 27.52 % and 37.65 % for DON and 79.66% and 83.30% for ZEN.

3.6 Influence of fermentation style on the reduction of mycotoxins in primary fermented wort

The characteristics of primary fermented wort with *Saccharomyces* yeasts at different temperatures are show in the Table 5.

The mycotoxin presence in the wort had no effect on the growth of *Saccharomyces* yeast used; consequently, it did not influence the alcohol production at the end of two fermentation beer styles. Lower fermentation temperatures resulted in lower DON adsorption in the yeasts. There was no significant difference between the DON reduction when the wort is fermented with S. *cerevisiae* compared to the fermentation by S. *pastorianus* strains. This could be because the reduction occurs mainly in the first 24h of the fermentation, which is the reason why the difference in fermentation times could not influence. Ale fermentation at 25 °C was the process with the highest ZEN decrease in wort and the highest content of it found in yeast residue. Low fermentation temperatures affect the adsorption of ZEN to *S. cerevisiae*, while there was no significant effect in the ZEN absorbed in *S. pastorianus* yeast after the fermentation.
Depending on fermentation variables, DON decrease in wort can be up to 14 %, while for ZEN up to 80 %. This reduction could explain the result of the studies of Pascari et al., 2018; Peters et al., 2017; Wall-Martínez, Pascari, Ramos, et al., 2019 who detected traces of ZEN and diverse concentrations of DON and its modified forms in beers of different styles in different countries. It is relevant to know the fermentation variables effect in the mitigation of mycotoxins because it can considerably reduce the mycotoxin intake for beer consumption.

4. Conclusion

The DON and ZEN decrease in yeast and fermented wort with different conditions process was analyzed. Mycotoxins concentration in wort had no effect on the ethanol production, growth rate of yeasts or DON and ZEN degradation during fermentation. In addition to the yeast growth and alcohol production, for DON, the fermentation temperature had an influence on mycotoxin decrease in wort and mycotoxin adsorption to the yeast. For ZEN, temperature only had influence in the mycotoxin adsorption on yeast. A second fermentation contributes to the reduction of mycotoxin levels in similar proportions as the first fermentation. Regarding the fermentation style, ale fermentation favored the adsorption of ZEN to *S. cerevisiae* cells and lager fermentation favored the adsorption of DON to *S. pastorianus* cells. The choice of adequate parameters is relevant to decrease the mycotoxin content during beer fermentation. A second fermentation would be a real option to decrease the mycotoxin content in previously fermented wort, ensuring that the yeast with adsorbed mycotoxin is removed from the final product. The use of QuEChERS as a method to extract mycotoxins obtained retention percentages similar to those obtained by immunoaffinity columns, with some advantages such as shorter extraction time, lower supplies cost, in addition to requiring a single extraction for both mycotoxins (DON and ZEN).

5. References

Andrews, S., Pardoel, D., Harun, A., & Treloar, T. (1997). Chlorine inactivation of fungal spores on cereal grains. *International Journal of Food Microbiology*, *35*(2), 153–162. https://doi.org/10.1016/S0168-1605(96)01214-7

Campagnollo, F. B., Franco, L. T., Rottinghaus, G. E., Kobashigawa, E., Ledoux, D. R., Daković, A., & Oliveira, C. A. F. (2015). In vitro evaluation of the ability of beer fermentation residue containing *Saccharomyces cerevisiae* to bind mycotoxins. *Food Research International*, *77*, 643–648. https://doi.org/10.1016/j.foodres.2015.08.032

Charoenchai, C., Fleet, G. H., & Henschke, P. A. (1998). Effects of temperature, pH, and sugar concentration on the growth rates and cell biomass of wine yeasts. *American Journal of Enology and Viticulture*, *49*(3), 283–288.

European Commission. (2006). Commission Regulation (EC) No 1881/2006 of 19 December 2006 setting

maximum levels for certain contaminants in foodstuffs. *Official Journal of the European Communitites*, *L364*(1881), 5–24. https://doi.org/10.2203/dose-response.06-012.Hanekamp

Garda, J., Martins Macedo, R., Faria, R., Bernd, L., Dors, G. C., & Badiale-Furlong, E. (2005). Alcoholic fermentation effects on malt spiked with trichothecenes. *Food Control*, *16*(5), 423–428. https://doi.org/10.1016/j.foodcont.2004.05.001

Huwig, A., Freimund, S., Käppeli, O., & Dutler, H. (2001). Mycotoxin detoxication of animal feed by different adsorbents. *Toxicology Letters*, *122*(2), 179–188. https://doi.org/10.1016/S0378-4274(01)00360-5

Keller, L., Abrunhosa, L., Keller, K., Rosa, C. A., Cavaglieri, L., & Venâncio, A. (2015). Zearalenone and its derivatives α-zearalenol and β-zearalenol decontamination by *Saccharomyces cerevisiae* strains isolated from bovine forage. *Toxins*, *7*(8), 3297–3308. https://doi.org/10.3390/toxins7083297

Kunze, W. (2006). *Tecnología para Cerveceros Y Malteros*. Retrieved from www.ame-kulessa.de

Lancova, K., Hajslova, J., Poustka, J., Krplova, A., Zachariasova, M., Dostalek, P., & Sachambula, L. (2008). Transfer of *Fusarium* mycotoxins and 'masked' deoxynivalenol (deoxynivalenol-3-glucoside) from field barley through malt to beer. *Food Additives & Contaminants: Part A*, *25*(6), 732–744. https://doi.org/10.1080/02652030701779625

Mizutani, K., Nagatomi, Y., & Mochizuki, N. (2011). Metabolism of zearalenone in the course of beer fermentation. *Toxins*, *3*(2), 134–141. https://doi.org/10.3390/toxins3020134

Nathanail, A. V., Gibson, B., Han, L., Peltonen, K., Ollilainen, V., Jestoi, M., & Laitila, A. (2016). The lager yeast *Saccharomyces pastorianus* removes and transforms *Fusarium* trichothecene mycotoxins during fermentation of brewer's wort. *Food Chemistry*, *203*, 448–455. https://doi.org/10.1016/j.foodchem.2016.02.070

Pascari, X., Gil-Samarra, S., Marín, S., Ramos, A. J., & Sanchis, V. (2019). Fate of zearalenone, deoxynivalenol and deoxynivalenol-3-glucoside during malting process. *Lwt*, *99*, 540–546. https://doi.org/10.1016/j.lwt.2018.10.030

Pascari, X., Ortiz-Solá, J., Marín, S., Ramos, A. J., & Sanchis, V. (2018). Survey of mycotoxins in beer and exposure assessment through the consumption of commercially available beer in Lleida, Spain. *Lwt*, *92*, 87–91. https://doi.org/10.1016/j.lwt.2018.02.021

Pascari, X., Rodriguez-Carrasco, Y., Juan, C., Mañes, J., Marin, S., Ramos, A. J., & Sanchis, V. (2019). Transfer of *Fusarium* mycotoxins from malt to boiled wort. *Food Chemistry*, *278*, 700–710. https://doi.org/10.1016/j.foodchem.2018.11.111

Perestrelo, R., Silva, P., Porto-Figueira, P., Pereira, J. A. M., Silva, C., Medina, S., & Câmara, J. S.

(2019). QuEChERS - Fundamentals, relevant improvements, applications and future trends. *Analytica Chimica Acta*, (xxxx). https://doi.org/10.1016/j.aca.2019.02.036

Peters, J., Van Dam, R., Van Doorn, R., Katerere, D., Berthiller, F., Haasnoot, W., & Nielen, M. W. F. (2017). Mycotoxin profiling of 1000 beer samples with a special focus on craft beer. *PLoS ONE*, *12*(10), 1–27. https://doi.org/10.1371/journal.pone.0185887

Rodríguez-Carrasco, Y., Fattore, M., Albrizio, S., Berrada, H., & Mañes, J. (2015). Occurrence of *Fusarium* mycotoxins and their dietary intake through beer consumption by the European population. *Food Chemistry*, *178*(1881), 149–155. https://doi.org/10.1016/j.foodchem.2015.01.092

Shetty, P. H., & Jespersen, L. (2006). *Saccharomyces cerevisiae* and lactic acid bacteria as potential mycotoxin decontaminating agents. *Trends in Food Science and Technology*, *17*(2), 48–55. https://doi.org/10.1016/j.tifs.2005.10.004

Souza-Silva, É. A., Gionfriddo, E., & Pawliszyn, J. (2015). A critical review of the state of the art of solid-phase microextraction of complex matrices II. Food analysis. *TrAC Trends in Analytical Chemistry*, *71*, 236–248. https://doi.org/10.1016/J.TRAC.2015.04.018

Torija, M. J., Rozès, N., Poblet, M., Guillamón, J. M., & Mas, A. (2003). Effects of fermentation temperature on the strain population of *Saccharomyces cerevisiae*. *International Journal of Food Microbiology*, *80*(1), 47–53. https://doi.org/10.1016/S0168-1605(02)00144-7

Vila-Donat, P., Marín, S., Sanchis, V., & Ramos, A. J. (2018). A review of the mycotoxin adsorbing agents, with an emphasis on their multi-binding capacity, for animal feed decontamination. *Food and Chemical Toxicology*, *114*, 246–259. https://doi.org/10.1016/j.fct.2018.02.044

Wall-Martínez, H. A., Pascari, X., Bigordà, A., Ramos, A. J., Marín, S., & Sanchis, V. (2019). The fate of *Fusarium* mycotoxins (deoxynivalenol and zearalenone) through wort fermenting by *Saccharomyces* yeasts (S. cerevisiae and S. pastorianus). *Food Research International*, *126*, 108587. https://doi.org/10.1016/j.foodres.2019.108587

Wall-Martínez, H. A., Pascari, X., Ramos, A. J., Marín, S., & Sanchis, V. (2019). Frequency and levels of mycotoxins in beer from the Mexican market and exposure estimate for deoxynivalenol mycotoxins. *Mycotoxin Research*, *35*(2), 207–216. https://doi.org/10.1007/s12550-019-00347-x

Table

Table 1. Validation parameters for the UHPLC/QuEChERS method for mycotoxins analysis in wort

Mycotoxin	Spiking levels, µg/kg	Replicates	Recovery ± SD (%)	Inter day precision RSD (%)
DON	75	5	81.22 ± 8.00	6.50
DON	250	7	73.69 ± 4.52	3.33
DON	1000	5	83.87 ± 4.92	4.77
ZEN	75	5	85.63 ± 11.44	9.80
ZEN	250	7	84.44 ± 11.60	9.72
ZEN	1000	5	90.71 ± 7.78	7.06

LOD DON: 20 µg/kg, LOD ZEN: 2.5 µg/kg.

SD: Standard deviation. RSD: Relative standard deviation.

Table 2. DON in *Saccharomyces* yeasts and wort fermented at different temperatures

Yeast code	Temperature (°C)	Initial mycotoxin	DON in wort (%)						Yeast (%)
			24 h	48 h	72 h	96 h	120 h	144 h	
A	15	Low	92.67 [a]	93.48 [a]	90.04 [a]	85.80 [a]	N A	N A	<LOD
A	15	High	92.85 [a]	94.38 [a]	86.91 [a]	85.04 [a]	N A	N A	<LOD
A	20	Low	84.08 [a]	88.82 [a]	93.25 [a+]	81.88 [a]	N A	N A	<LOD
A	20	High	85.47 [ab]	71.80 [b]	72.74 [b]	78.72 [b]	N A	N A	3.16 ± 0.99
A	25	Low	76.92 [ab]	67.30 [b]	76.61 [ab]	76.89 [ab]	N A	N A	<LOD
A	25	High	82.51 [ab]	70.29 [b]	65.87 [b]	72.49 [b]	N A	N A	2.13 ± 0.17
B	15	Low	97.12 [a]	89.46 [a]	91.23 [a]	87.45 [a]	N A	N A	<LOD
B	15	High	92.97 [a]	94.18 [a]	91.77 [a]	89.05 [a]	N A	N A	<LOD
B	20	Low	83.39 [ab]	85.75 [ab]	80.41 [ab]	75.38 [b]	N A	N A	<LOD
B	20	High	83.79 [a]	83.67 [a]	85.78 [a]	84.71 [a]	N A	N A	2.89 ± 0.49
B	25	Low	80.60 [b]	79.31 [b]	78.64 [b]	80.75 [b]	N A	N A	<LOD
B	25	High	81.98 [ab]	80.23 [b]	78.77 [b]	77.38 [b]	N A	N A	3.65 ± 0.73
C	10	Low	97.18 [a]	96.05 [a]	95.60 [a]	86.59 [a]	86.30 [a]	82.52 [a]	<LOD
C	10	High	93.80 [a]	92.58 [a]	92.46 [a]	88.09 [a]	87.82 [a]	85.34 [a]	<LOD
C	15	Low	95.66 [ab]	95.07 [ab]	88.91 [ab]	86.95 [ab]	79.26 [ab]	75.82 [b]	<LOD
C	15	High	93.66 [ab]	89.76 [ab]	82.89 [b]	82.94 [b]	81.42 [b]	81.42 [b]	4.08 ± 0.31
C	20	Low	92.53 [ab]	90.16 [ab]	86.79 [ab]	88.05 [ab]	81.52 [ab]	75.60 [b]	6.15 ± 0.24
C	20	High	92.34 [ab]	87.68 [ab]	84.24 [a]	80.87 [b]	79.00 [b]	78.18 [b]	5.03 ± 0.66
D	10	Low	96.45 [a]	95.81 [a]	94.08 [a]	88.53 [a]	87.09 [a]	85.54 [a]	<LOD
D	10	High	95.84 [a]	92.63 [a]	90.27 [a]	88.81 [a]	85.14 [a]	82.91 [a]	<LOD
D	15	Low	95.60 [a]	93.15 [ab]	84.22 [abc]	83.60 [abc]	77.81 [bc]	74.38 [c]	<LOD
D	15	High	90.70 [a]	91.06 [a]	84.22 [ab]	84.06 [ab]	81.65 [ab]	79.99 [b]	3.45 ± 0.40
D	20	Low	89.95 [ab]	87.50 [ab]	83.26 [ab]	81.94 [ab]	81.49 [ab]	76.70 [b]	<LOD
D	20	High	91.79 [ab]	90.57 [ab]	80.99 [ab]	79.59 [ab]	77.07 [b]	75.41 [b]	4.56 ± 0.30

Means of triplicate determinations

A, B - *S. cerevisiae* yeast and C, D - *S. pastorianus* yeast

[a-b] Levels with different letters in the same row are significantly different ($P < 0.05$)

Sample with mark (+) is significantly different ($P < 0.05$) to the sample with the same process parameters (yeast and temperature) but with high mycotoxin concentration.

Initial high contamination 1164 ± 57 µg/kg, Initial DON low contamination 560 ± 49 µg/kg

LOD DON 20 µg/kg

Table 3. ZEN concentration in *Saccharomyces* yeast and wort fermented at different temperatures

Yeast code	Temperature (°C)	Level of mycotoxin	ZEN in wort (%)						Yeast (%)
			24 h	48 h	72 h	96 h	120 h	144 h	
A	15	Low	86.66 ^{ab}	83.23 ^{ab}	81.56 ^{ab}	71.59 ^{b}	N A	N A	7.99 ± 2.73
A	15	High	88.30 ^{ab}	82.96 ^{b}	78.10 ^{bc}	69.50 ^{c}	N A	N A	5.49 ± 2.21
A	20	Low	48.81 ^{b}	23.13 ^{c}	15.83 ^{c}	24.01 ^{c}	N A	N A	28.43 ± 9.83
A	20	High	42.60 ^{b}	30.84 ^{b}	20.89 ^{b}	25.50 ^{b}	N A	N A	25.24 ± 4.05
A	25	Low	26.85 ^{b}	12.54 ^{c}	12.02 ^{c}	16.09 ^{c}	N A	N A	33.86 ± 3.07
A	25	High	41.47 ^{b}	13.70 ^{c}	12.46 ^{c}	9.74 ^{c}	N A	N A	31.97 ± 5.59
B	15	Low	108.00 ^{ab}	77.35 ^{abc}	67.25 ^{bc}	58.87 ^{c}	N A	N A	8.53 ± 2.41
B	15	High	102.83 ^{a}	88.33 ^{ab}	80.16 ^{ab}	66.57 ^{b}	N A	N A	5.41 ± 1.29
B	20	Low	45.98 ^{b}	32.74 ^{bc}	24.21 ^{c}	21.63 ^{c}	N A	N A	19.10 ± 2.71
B	20	High	44.71 ^{b}	49.64 ^{b}	41.05 ^{b}	32.54 ^{b}	N A	N A	24.16 ± 8.18
B	25	Low	44.06 ^{b}	24.01 ^{c}	16.38 ^{c}	15.92 ^{c}	N A	N A	28.55 ± 2.38
B	25	High	36.07 ^{b}	33.32 ^{b}	27.29 ^{b}	32.95 ^{b}	N A	N A	29.52 ± 3.18
C	10	Low	91.17 ^{ab}	90.92 ^{ab}	72.41 ^{ab}	62.19 ^{b}	59.51 ^{b}	60.54 ^{b}	9.89 ± 4.81
C	10	High	95.47 ^{a}	84.86 ^{a}	81.20 ^{a}	56.62 ^{b}	53.45 ^{b}	55.51 ^{b}	11.44 ± 4.08
C	15	Low	95.54 ^{a}	90.04 ^{a}	76.80 ^{ab}	56.48 ^{bc}	44.32 ^{c}	43.43 ^{c}	11.53 ± 3.11
C	15	High	96.55 ^{a}	81.65 ^{b}	72.65 ^{bc}	60.64 ^{cd}	48.02 ^{d}	45.56 ^{d}	15.63 ± 4.35
C	20	Low	78.45 ^{ab}	74.70 ^{ab}	69.08 ^{bc}	45.22 ^{cd}	39.63 ^{d}	41.11 ^{d}	23.57 ± 2.41
C	20	High	89.07 ^{ab}	84.96 ^{b}	66.40 ^{c}	53.62 ^{cd}	44.51 ^{d}	44.85 ^{d}	19.34 ± 5.37
D	10	Low	87.25 ^{ab}	70.71 ^{bc}	57.52 ^{cd}	45.85 ^{d}	50.45 ^{d}	47.44 ^{d}	9.85 ± 6.40
D	10	High	95.43 ^{a}	83.96 ^{a}	59.19 ^{a}	58.55 ^{b}	45.85 ^{b}	45.77 ^{b}	9.5 ± 0.67
D	15	Low	83.76 ^{b}	57.58 ^{c}	45.29 ^{cd}	45.38 ^{cd}	42.42 $^{d+}$	38.07 ^{d}	24.06 ± 0.95
D	15	High	76.22 ^{b}	50.75 ^{c}	41.60 ^{cd}	39.82 ^{cd}	27.56 $^{d+}$	36.85 ^{cd}	23.01 ± 3.30
D	20	Low	79.68 ^{b}	62.41 $^{c+}$	42.68 ^{d}	45.73 ^{d}	36.61 ^{d}	37.31 ^{d}	24.83 ± 1.74
D	20	High	73.25 ^{b}	46.63 $^{c+}$	35.41 ^{c}	41.50 ^{c}	33.37 ^{c}	33.70 ^{c}	19.12 ± 3.78

Means of triplicate determinations

A, B- *S. cerevisiae* yeast and C, D - *S. pastorianus* yeast

$^{a-d}$ Levels with different letters in the same row are significantly different ($P < 0.05$).

Sample with mark (+) is significantly different ($P < 0.05$) to the sample with the same process parameters (number of yeast and temperature) but with high mycotoxin concentration.

Initial ZEN high contamination was 440 ± 16 µg/kg Initial ZEN low contamination was 284 ±14 µg/kg.

LOD ZEN 2.5 µg/kg.

Table 4. ZEN and DON after secondary fermentation and storage of beer

Mycotoxin	Mycotoxin in wort after double fermentation (%)	Mycotoxin in yeast (%)	Mycotoxin during storage				Total Reduction (first, second fermentation and storage) (%)
			1st week (%)	2nd week (%)	3th week (%)	4th week (%)	
DON	84.87 [a]	5.12 ± 2.73	84.53 [a]	87.12 [a]	90.22 [a]	88.73 [a]	27.52 ± 5.24
	85.43 [a]	4.78 ± 0.44	79.81 [a]	79.22 [a]	82.50 [a]	76.70 [a]	37.65 ± 6.29
ZEN	49.01 [a]	27.17 ± 5.54	51.45 [a]	39.10 [b]	45.61 [ab]	47.38 [a]	83.30 ± 3.87
	63.98 [a]	21.43 ± 3.22	61.00 [a]	66.56 [a]	55.80 [a]	57.72 [a]	79.66 ± 4.21

Means of triplicate determinations
Levels with different letters in the same row are significantly different ($P < 0.05$).
Initial DON contamination 950.76 ± 12 µg/kg. Initial ZEN contamination 197.34 ± 5 µg/kg
LOD DON 20 µg/kg. LOD ZEN 2.5 µg/kg.

Table 5. Characteristics of yeasts and wort fermented with *Saccharomyces* yeasts at different temperatures

Fermentation style	Fermentation temperature (°C)	UFC final		Ethanol produced in the wort (%)		DON adsorbed on yeast (%)	DON in wort after fermentation (%)	ZEN adsorbed on yeast (%)	ZEN in wort after fermentation (%)
		Control	Contaminated	Control	Contaminated				
Ale (*S. cerevisiae*)	15	10^8	10^8	3.41 [a]	3.42 [a]	<LOD	86.83 [a]	6.86 [a]	66.63 [a]
	20	10^9	10^9	3.92 [b]	3.90 [b]	3.02 [b]	78.57 [ab]	24.23 [b]	23.94 [c]
	25	10^9	10^9	4.19 [c]	4.11 [c]	2.89 [b]	76.88 [b]	30.97 [c]	18.13 [c]
Lager (*S. pastorianus)*	10	10^8	10^8	2.79 [d]	2.71 [d]	<LOD	84.08 [ab]	10.17 [a]	47.38 [b]
	15	10^8	10^8	3.47 [a]	3.51 [a]	3.93 [ab]	77.90 [ab]	18.56 [b]	41.82 [b]
	20	10^9	10^9	3.43 [a]	3.47 [a]	5.02 [a]	76.47 [b]	21.71 [b]	39.79 [b]

UFC initial 10^6 for control and contaminated wort

[a-c] Levels with different letters in the same column are significantly different ($P < 0.05$).

Level with mark (+) is significantly different ($P < 0.05$) with control sample

LOD ZEN 2.5 µg/kg, LOD DON 20 µg/kg